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ORIGINAL ARTICLE

The significance of DNA image cytometry and Edmondson-Steiner grading on prognosis after curative resection of hepatocellular carcinoma

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Summary

Purpose: *Hepatocellular carcinoma (HCC) is the commonest primary cancer of the liver. Hepatic resection remains the main curative option, although the incidence of disease recurrence in the remaining hepatic parenchyma is high and accounts for the leading cause of death post resection. For this reason, the need to identify prognostic factors which may determine treatment response and survival is of paramount importance. In this study we assessed whether DNA image cytometry and Edmondson-Steiner grading could be used as prognostic factors in a cohort of patients with HCC undergoing radical hepatic resection.*

Methods: *Forty-four patients with HCC who underwent radical resection were retrospectively analyzed. Histological grading according to Edmondson and Steiner and DNA ploidy using DNA image cytometry, were the two parameters analyzed. Pearson's x^2 or Fisher's exact tests were used to test for any associations between categorical variables. Univariate semi-parametric Cox proportional hazard regression models were used to assess the effect of explanatory variables on death. All reported p values were based on two-sided tests and compared to a significance level of 0.05.*

Results: *In univariate Cox regression analysis, adverse survival outcome was strongly associated with high DNA score and advanced histological grading. Patients with ploidy score >2.2 had 3.95 times higher probability of death, as compared to those with ploidy score ≤2.2. Edmondson-Steiner grades III and IV were also associated with 20.49 and 34.47 higher probability of death respectively as compared to grade I.*

Conclusion: *Our results validate the prognostic significance of DNA image cytometry and Edmondson-Steiner grading following curative resection of HCC.*

Key words: cancer, cytometry, DNA, hepatocellular carcinoma

Introduction

HCC is the commonest primary cancer of the liver. Incidence is increasing and HCC is currently the 5th commonest malignancy worldwide and the 3rd leading cause of cancer-related deaths following lung and gastric cancer. The burden from HCC is likely to increase in the foreseeable future with increasing rates of alcoholism, hepatitis B and C prevalence, and obesity-related fatty liver disease; a concurrent shift in incidence towards younger persons is eminent [1]. Resection, ablation, and orthotopic liver transplantation (OLT) have all been proposed for treating HCC, however the subsets of patients who benefit most from each modality remain controversial [2]. Hepatic resection remains the main curative option for this malignancy, especially for patients with small tumors; however, the incidence of disease recurrence in the remaining hepatic parenchyma is high, accounting for the leading cause of death after surgical resection of HCC [2,3]. Advances in intraoperative techniques and postoperative care have led to a marked decline in perioperative death rates and complications after hepatic resection for HCC. Nevertheless and in view of the emerging availability of alternative treatment modalities, the need to identify prognostic factors which may determine treatment response and survival on an individual basis becomes compelling. In this study we focused on cell morphom-

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etry and we sought to identify whether DNA image cytometry and Edmondson-Steiner grading could be used as prognostic factors in a cohort of patients with HCC undergoing radical hepatic resection.

Methods

Patients and data sources

A total of 44 selected patients with HCC who underwent radical resection and had at least one follow up review were retrospectively analyzed. Selection was based in accordance to the following criteria: 1) R0 resection status; 2) solitary or multiple tumors/no more than 3, involving one lobe (38 patients with solid tumor, and 5 patients with multiple unilobar tumors); 3) tumors < 8 cm in diameter (41 tumors < 5 cm, and no more than 3 tumors > 5 cm but < 8 cm); 4) no evidence of tumor thrombus in the main trunk of portal vein; 5) no evidence of extrahepatic metastatic disease (including hepatoduodenal lymph node involvement, invasion of adjacent anatomical structures/e.g. diaphragm, etc.); 6) anatomical liver resections (non-typical and wedge resections were excluded); 7) Child-Pugh grading no worse than grade B; and 8) absence of significant comorbidities (particularly severe ischaemic heart disease and chronic obstructive lung disease). *Post hoc* power analysis showed that the sample size of 44 patients was adequate to reveal real differences in the proportions higher than 30%, achieving statistical power of 79% at a significance level of 0.05. Patients were hepatectomized between August 1997 and May 2005; all had curative resection for HCC in the 1st Surgical Department of "Laiko" hospital, Athens, Greece. Curative hepatectomy was defined by the absence of visible remnant lesions and/or lesions demonstrable during an intraoperative examination of the residual liver. Thirty-six patients had major resections including 24 right hepatectomies and 12 left hepatectomies, and 8 patients had typical segmentectomies including 5 right posterior hepatectomies and 3 left lateral hepatectomies. Operative mortality as defined by death within 45 days of hepatic resection was 0%. Two parameters were selected for analysis: histological grading according to Edmondson and Steiner and DNA ploidy. Histological grading according to Edmondson-Steiner criteria [4] showed that patients with grades I, II, III and IV numbered 10 (22.7%), 12(27.3%), 9 (20.5%), and 13 (29.5%), respectively. For DNA ploidy, group categorisation was applied for analytical purposes. Among the 44 tumors analysed 31 (70.5%) exhibited a ploidy score of ≤ 2.2 and 13 (29.5%) had a ploidy score of >2.2.

DNA measurements (ploidy)

DNA image cytometry and DNA index/ploidy score calculation were performed as previously described [5]. Briefly, the nuclei of Feulgen-stained cells were evaluated for DNA ploidy using a Nikon eclipse microscope (Nikon, Japan) connected with a Nikon CCD videocamera and an IBM Pentium 4/PC with the appropriate Cell Measurement Software (Image Pro Plus v. 5.1, Media Cybernetics Inc, Silver Springs, MD, USA). A total of 100-200 nuclei with clear boundaries appearing to have no loss of membrane integrity were identified for analysis from each tissue sample. Measurements were made using a magnification of ×200. This analysis configuration permits operator-dependent selection and measurement of DNA content. This cell measurement system was calibrated before each analysis session using a slide with human normal lymphocytes with known DNA content. The data generated were downloaded to standard software packages for final analysis. DNA histograms were categorized as diploid if the histogram presented a single peak (2c; c=haploid DNA content) in the G0-G1 area and the cell nuclei population did not exceed 10% in the G2 region (4c). A sample was considered aneuploid if clear aneuploid peaks (3c, 5c, 7c and 9c) were present. A DNA index/ploidy score between 0.9 and 1.1 was considered diploid, aneuploid 1.1-1.4, triploid 1.4-1.8, tetraploid 1.8-2.2, hypertetraploid >2.2. For each case, coefficient of variance (CV) and DNA index /ploidy score was calculated relative to internal controls (lymphocytes; DI= 0.1).

Statistical analysis

Continuous variables are presented as mean values ± standard deviations, while categorical ones are presented as absolute and relative frequencies (percentages). The Shapiro-Wilk criterion was used for the assessment of normality. All variables exhibited a normal distribution, and therefore classic statistical tests were used. Pearson's x^2 or Fisher's exact tests were used in order to test for any associations between two categorical variables. The ploidy continuous variable was transformed to a categorical one, based on the cut-off value of 2.2. Death was estimated using the Kaplan-Meier method and the patients were censored at the date of death. The effect of the explanatory variables on death was evaluated using univariate semi-parametric Cox proportional hazard regression models. All reported p values were based on two-sided tests and compared to a significance level of 0.05. Data were analyzed using STATA™ statistical software (Version 9.0, Stata Corporation, College Station, TX 77845, USA).

Results

Patients

A total of 44 patients were included in the study, with mean age of 50.5 years and standard deviation of 10.5 years. The clinical characteristics are shown in Table 1. Ploidy (both as a categorical and as a continuous variable), grade and follow-up time differed significantly between survived and deceased patients. The majority of survived patients had a ploidy < 2.2, compared to the deceased ones (90.5 vs. 52.2% respectively, p=0.005). There was a significantly higher proportion of grade I and II in the survived group relative to the deceased group (90.5 vs. 13.0% respectively, p <0.001).

Survival analysis

Survival data were collected for all patients. The mean follow up time was 41.9 months. Based on the Kaplan-Meier method, the median survival time was recorded at 52 months (range 3-91).

Hazard ratios of risk factors

In univariate analysis there were significant differences in survival among the groups stratified according to DNA ploidy and Edmondson-Steiner grading (Table 2). A high DNA content was found to be associated with adverse prognosis as patients with ploidy score >2.2 had 3.95 times higher probability of death, as compared to those with ploidy score ≤ 2.2 (Figure 1). Edmondson-Steiner grading was also identified as predictive for survival grades III and IV, which were associated with 20.49 and 34.47 higher probability of death as compared to grade I (Figure 2).

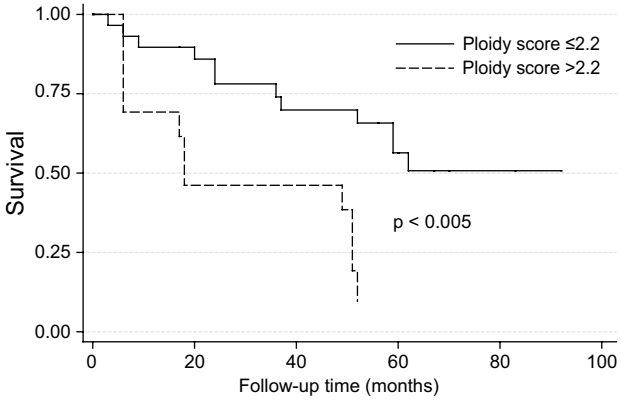

Figure 1. Univariate analysis of survival rates of ploidy score ≤2.2 and ploidy score >2.2.

Table 1. Clinical characteristics of the sample

		Survived		*Deceased*			*Total*	
Characteristics	*Category*	*N*	*%*	*N*	*%*	*p-value*	*N*	*%*
Ploidy	<2.2	19	90.50	12	52.20	0.005	31	70.50
	>2.2	2	9.50	11	47.80		13	29.50
Grade	I	9	42.90	1	4.30	< 0.001	10	22.70
	II	10	47.60	2	8.70		12	27.30
	III	1	4.80	8	34.80		9	20.50
	IV	1	4.80	12	52.20		13	29.50
		Mean	*SD*	*Mean*	*SD*	*p-value*		
Ploidy value		1.76	0.33	2.17	0.324	< 0.001	1.98	0.383
Follow-up time (months)		57.32	24.53	29.17	20.96	< 0.001	41.9	26.48

SD: standard deviation

Table 2. Univariate Cox proportional hazard regression model

Variable	Hazard ratio	95% confidence interval		p-value
		Lower	*Upper*	
Ploidy score >2.2 vs. < 2.2	3.95	1.61	9.68	0.003
Grade II vs. I	3.23	0.29	35.82	0.339
Grade III vs. 1	20.49	2.51	166.92	0.005
Grade IV vs. I	34.47	4.27	278.12	0.001

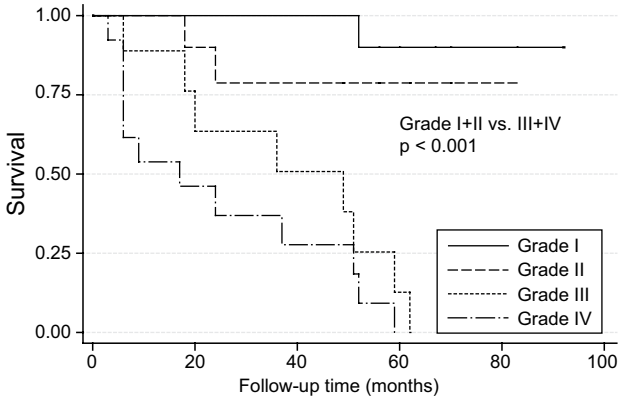

Figure 2. Univariate analysis of survival rates according Edmondson-Steiner grades I-IV.

Discussion

Our study has shown high DNA content (high ploidy score or index) to be associated with worst prognosis in HCC patients following curative resection. Evidence is accumulating that DNA image cytometry has prognostic significance in many malignancies but relatively few studies have examined the prognostic significance of ploidy in HCC with conflicting results. Among these studies, 8 have clearly demonstrated the prognostic significance of DNA ploidy in HCC patients [6-13], whereas 2 have failed to do so [14,15]. DNA ploidy in the adult human liver is difficult to interpret as a certain degree of polyploidisation is physiological. Normal human liver has been found to contain 10-40% polyploid cells, 10-15% of which are binucleate and these cells are thought to represent the end stage of differentiation and could be interpreted as a sign of increased proliferative capacity [16,17]. Further discrepancies have been attributed to the technique employed with the majority of published studies using flow cytometric analysis of DNA even though DNA image cytometry is considered a superior technique for the detection of DNA ploidy abnormalities. Its advantage over flow cytometry relies on the fact that only tumor cells are used for DNA measurement since it allows direct microscopic visualisation of the biopsy material and selection of tumor cells for analysis [18,19]. Further to this, set-up costs are low and it is routinely performed on formalin-fixed paraffin-embedded samples which allows analysis of archival material which is valuable when planning longitudinal studies on disease progression [20]. Despite its advantages DNA image cytometry has had limited application as it has been used in 2 of the 10 published studies examining the prognostic significance of DNA ploidy in HCC. Both these two studies have shown clear associations between DNA ploidy and survival outcome in HCC following surgical resection conforming to our results [12,13].

Our study has also demonstrated that the Edmondson-Steiner grading system which reflects the differentiation grade of the tumor on the basis of cellular characteristics such as size, morphology, and mitotic figures, has a strong impact on survival as advanced grades (grade III and IV) were shown to be associated with dismal prognosis. This also conforms to previous studies validating the prognostic significance of HCC differentiation status in patients undergoing various treatments including resection [20-25], orthotopic liver transplantation [26], radiofrequency ablation, percutaneous ethanol or acetic acid injection and microwave coagulation [27-29]. As the prognostic significance of histological grading is not universally accepted, it has not been incorporated in commonly used staging systems including the widely accepted Tumor-Node-Metastasis (TNM) staging by the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC). This mainly considers clinicopathological parameters for T classification such as the number of tumor nodules, the size of the largest nodule, and the presence or absence of blood vessel invasion [30]. Nevertheless Zhou et al. have shown by multivariate analysis Edmondson-Steiner grading and not TNM-5 or TNM-6 to be of independent significance for survival in their cohort of 171 patients with HCC. Further to this, it was suggested that a novel prognostic scoring system integrating Edmondson-Steiner grading and TNM-6 could be of stronger predictive value in curatively resected HCC [23].

Conclusions

In conclusion, DNA ploidy and histological grading according to the Edmondson-Steiner criteria may provide a reliable, objective indicator of tumor biology that may identify patients with HCC who are more likely to benefit from surgical resection. The study is limited by its retrospective design and the small patient cohort, however its results highlight the importance of considering DNA ploidy and Edmondson-Steiner grading in the selection of appropriate management strategies for HCC patients and the design of future clinical trials.

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